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Chemotherapeutic Agents

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INTRODUCTION

The general subject of this research project is the effects of dietary supplements on the pharmacokinetics and pharmacodynamics of chemotherapeutic drugs used to treat women with breast cancer. More specifically, the research focuses on the effects of St. John's wort as an example of a nutraceutical and Vitamin E as an example of a nutritional supplement on doxorubicin, docetaxel, and cyclophosphamide. The hypothesis to be tested is that nutritional supplements have important effects on the pharmacokinetics and pharmacodynamics of cancer chemotherapeutic agents. The purpose of the research is to better understand the interaction of dietary supplements with cancer chemotherapeutic drugs and then utilize this knowledge to alert patients and their physicians to these interactions. This information also may be useful to decrease the toxicity and increase the effectiveness of chemotherapeutic drugs. The scope of the research involves *in vivo* assessments in rats of nutritional supplement-chemotherapeutic drug interactions and *in vitro* studies of the mechanisms of nutraceutical-chemotherapeutic drug interactions.

BODY

Task 1. Evaluate the effects of supplementation with St. John's wort and vitamin E on the pharmacokinetics of cyclophosphamide, docetaxel and doxorubicin.

- a. Establish and refine, as necessary, methodology for analysis of plasma concentrations of 4-hydroxycyclophosphamide, docetaxel, doxorubicin, vitamin E and hypericin.
 - b. Maintain 3 groups of rats on diet alone, or diet plus St. John's wort or vitamin E.
 - c. Inject rats with chemotherapeutic agents and collect plasma samples.
 - d. Measure drug levels in plasma.
 - e. Analyze pharmacokinetic data.
 - f. Repeat pharmacokinetic studies with different doses.
- a. Establish and refine, as necessary, methodology for analysis of plasma concentrations of 4-hydroxycyclophosphamide, docetaxel, doxorubicin, vitamin E and hypericin.**
- i. As reported previously, we have refined the technique to measure α -Tocopherol (vitamin E) concentrations in plasma using a reverse-phase high-performance liquid chromatography method with UV detection as previously reported by Julianto *et al.* (1). We used this method to measure vitamin E levels in rats maintained on diets with differing vitamin E contents.
 - ii. As reported previously, our original plan was to focus on analysis of hypericin as an indicator molecule for our pharmacological studies of St. John's wort. However, because some recent studies have identified hyperforin as a more important contributor to the pharmacological actions of this herbal product (2), we have employed an HPLC analytical method that accurately detects and determines hyperforin.
 - iii. As reported previously, doxorubicin concentrations in plasma were measured with a reverse-phase high-performance liquid chromatography method using fluorescence detection as previously reported by Warren *et al.* (3) and as modified by us.
 - iv. The method for assaying plasma docetaxel concentrations was refined using a modification of the method described by Parise *et al.* (4). Docetaxel concentrations in plasma were measured

with a reverse-phase high-performance liquid chromatography with UV detection. Briefly, docetaxel was extracted from plasma by solid-phase extraction on 1mL Sep-Pak CN columns (Waters, Milford, MA). The columns were first conditioned with 2 1mL aliquots of methanol followed by 2 1mL aliquots of 0.01M ammonium acetate (pH 5.0). Samples were loaded onto individual columns and washed with 2 1mL aliquots of 0.01M ammonium acetate, 2 1mL aliquots of 20% methanol in 0.01M ammonium acetate (pH 5.0), and 1mL hexane. Docetaxel was eluted from the columns using 1mL of acetonitrile. The eluents were evaporated to dryness under nitrogen and the residues were reconstituted in mobile phase. The mobile phase consisted of 45% acetonitrile in water with a flow rate of 1.0mL/min through an Econosphere C18 5 μ column with a matching guard column (Alltech, Deerfield, IL). The detector was operated at a wavelength of 227nm and the samples were quantified using peak height.

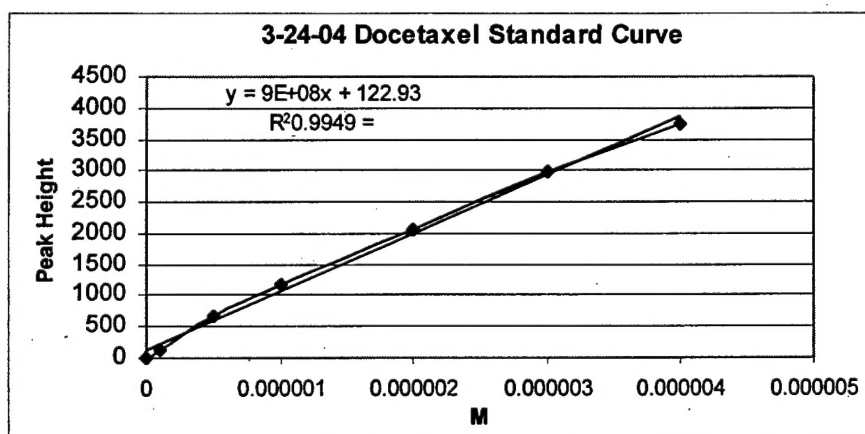


Figure 1. Docetaxel Standard Curve

- b. **Maintain 3 groups of rats on diet alone, or diet plus St. John's wort or vitamin E.**
 - i. As reported previously, weanling female Fisher 344 rats, 4 or 5 rats per group, were fed either a cereal-based, standard rat diet that supports growth and maintenance (Harlan-Teklad Global, Product # TD 00217) that contains 16% protein, 3.5% fat and 102 I.U./kg Vitamin E (α -tocopherol), or the same diet supplemented with either a high level of vitamin E, 750 I.U./kg (Harlan-Teklad Product # T.D. 01375) or a low level of vitamin E, 50 I.U./kg (Harlan-Teklad Product # T.D. 01374). The plasma levels of vitamin E were measured and confirmed that dietary vitamin E content influenced plasma vitamin E levels. Thus, rats on the diet containing 102 I.U./kg vitamin E had plasma levels of 15.4 μ M, those on the diet containing 152 I.U./kg vitamin E had plasma levels of 20.0 μ M, and those on the diet containing 750 I.U./kg vitamin E had plasma levels of 26.2 μ M. These values are similar to levels reported in the literature for rodents maintained on diets enriched for vitamin E (5).
 - ii. As previously reported, initial exploratory studies were performed with a standardized preparation of St. John's wort (HBC St. Johns wort) that was used in clinical trials and pharmacokinetic studies supported by the National Institutes of Health. A suspension was administered daily to rats by gavage. Unfortunately, all of the animals lost weight, and most died before the completion of a planned 14 day course. A review of the ingredients of this St. John's wort preparation indicated that the excipients included silicon dioxide. We believe that

this ingredient caused intestinal complications. However, several other St. John's wort preparations also caused toxicity when given by gavage. Further exploratory studies suggested that the stress of gavage in young rats contributed importantly to the observed mortality. Therefore we incorporated the St. John's wort into the diet rather than administer it by gavage. A custom diet was formulated that consists of Teklad Global 16% Protein Rodent Diet with 4 g of St. John's wort /kg of feed (4 mg/g). The St. John's wort preparation is Optical Nutrients product 14772 which consists of flowering tops and leaves and is standardized to 0.3% hypericin. Other ingredients include maltodextrin, rice powder, gelatin and magnesium stearate. A 150g rat ingests about 15 g of chow per day or 400mg/kg of body weight. This quantity of dietary St. John's wort has been calculated to approximate pharmacologically relevant doses of St. John's wort in humans (6). On this diet the rats grow at the same rate and ingest the same quantity of food as rats maintained on a control diet. The hematocrit and white blood cell counts measured after 2 weeks on the diet are not significantly different from rats maintained on the standard diet. After 2 weeks on the diet, the rats were found to have hyperforin levels of $2.50 \pm 0.69 \times 10^{-6}$ M. This drug concentration is comparable to levels we measured in rats that were given St. John's wort by gavage ($1.38 \pm 0.32 \times 10^{-6}$ M) and similar to levels of hyperforin reported in the literature by other laboratories (6). A lower dietary intake of 1 g of St. John's wort/kg of feed (1 mg/g) resulted in a hyperforin level of $2.04 \pm 0.69 \times 10^{-6}$ M.

- c. **Inject rats with chemotherapeutic agents and collect plasma samples.** As reported previously, all samples were obtained from the saphenous vein of the animals. Both lower hind legs on each animal were shaved. A thin layer of silicone grease was applied, and the leg was held with sufficient pressure to cause the vein to become clearly visible. Using a Microlance blood lancet a small puncture wound was made in the saphenous vein. The blood was collected in a StatSpin heparinized collection tube, mixed, and centrifuged at 12,000 rpm for 10 minutes. The plasma was removed, placed in microcentrifuge vials and stored at -80°C until testing. Blood samples were obtained prior to injection of the drug and at 10, 20 and 30 min, and 1, 3, 5, 7, 24 and 48 hrs.

d. **Measure drug levels in plasma.**

- i. Pharmacokinetic parameters of doxorubicin in control, vitamin E or St. John's wort-treated rats was presented in last year's annual report.

- ii. Pharmacokinetics of docetaxel in control, vitamin E or St. John's wort-treated rats.

Animals were injected with 10mg/kg of docetaxel. Blood samples from rats on the control diet, the vitamin E supplemented diets, and the St. John's wort supplemented diet have been collected and frozen. Initial results are illustrated in Table 1, below.

We have collected two sets of samples for pharmacokinetic analyses. In the first set, we used reconstituted powdered docetaxel. As illustrated below in Table 1, we got unsatisfactory values, with unmeasurable early time points despite IV administration of the drug. We considered that either 1) we needed to draw earlier time points, 2) we were having precipitation of the reconstituted drug or 3) our detection system was not sensitive enough. Therefore a second set of samples was collected using the commercially available drug, with earlier time points. We also asked Dr. Merrill Egorin at the University of Pittsburgh to run a set of samples, since we are using UV detection and he is using mass spectroscopy. His more sensitive detection system could measure docetaxel in the early time points. Therefore Dr. Egorin will be running all (over 500) of our pharmacokinetic samples (see Description of Experiments, below).

Table 1. Docetaxel pharmacokinetics.

Group 13518 Vitamin E-Docetaxel Pharmacokinetic Summary

Control Diet (102 IU Vitamin E) treated with 10 mg/kg Docetaxel

13518-1 24-Sep-03	Time [min]	Retention Time	Peak Height	[M]
	0		0	0
	10		0	0
	20		0	0
	30		0	0
	60		0	0
	180	9.9	142	5.12x10 ⁻⁷
	300	9.9	181	6.09x10 ⁻⁷
	420	9.9	147	5.24x10 ⁻⁷
	1440		0	0
	2880		0	0

13518-2 23-Mar-04	Time [min]	Retention Time	Peak Height	[M]
	0		0	0
	10		0	0
	20		0	0
	30	8.78	44	0
	60		0	0
	180	8.64	332	1.85x10 ⁻⁷
	300	8.66	374	2.27x10 ⁻⁷
	420	8.66	456	3.09x10 ⁻⁷
	1440	8.9	345	1.98x10 ⁻⁷
	2880	8.68	238	9.10x10 ⁻⁸

13518-3 23-Mar-04	Time [min]	Retention Time	Peak Height	[M]
	0		0	0
	10	8.66	1076	9.29x10 ⁻⁷
	20		0	0
	30		0	0
	60	8.66	94	0
	180	8.64	345	1.98x10 ⁻⁷
	300	8.64	191	4.40x10 ⁻⁸
	420	8.64	221	7.40x10 ⁻⁸
	1440		0	0
	2880		0	0

13518-4 24-Mar-04	Time [min]	Retention Time	Peak Height	[M]
	0		0	0
	10		0	0
	20		0	0
	30		0	0
	60		0	0
	180	9.4	159	4.01x10 ⁻⁸
	300	9.34	217	1.05x10 ⁻⁷
	420	9.37	349	2.51x10 ⁻⁷
	1440	9.39	133	1.12x10 ⁻⁸
	2880		0	0

Group 13518 Vitamin E-Docetaxel Pharmacokinetic Summary

Control Diet (102 IU Vitamin E) treated with 10 mg/kg Docetaxel

13518-5	Time [min]	Retention Time	Peak Height	[M]
24-Mar-04	0		0	0
	10		0	0
	20		0	0
	30	9.34	109	0
	60	9.4	313	2.11x10-7
	180	9.38	307	2.05x10-7
	300	9.39	584	5.12x10-7
	420	9.38	279	1.73x10-7
	1440		0	0
	2880		0	0

13518-6	Time [min]	Retention Time	Peak Height	[M]
25-Mar-04	0		0	0
	10		0	0
	20		0	0
	30		0	0
	60	9.25	455	3.12x10-7
	180	9.24	215	4.55x10-7
	300	9.21	328	1.71x10-7
	420	9.21	258	9.33x10-8
	1440		0	0
	2880		0	0

Descriptions of the experiments.

Vit.E/Docetaxel Pharmacokinetics

F344 rats

Diets from Harlan Teklad

6 animals on Control Diet

6 animals on Low E Diet (control diet plus 50IU Vit.E)

6 animals on High E Diet (control diet plus 150IU Vit.E)

Animals remained on diets for eight weeks prior to receiving a 10mg/kg dose of docetaxel i.v.
Heparinized blood samples were collected via saphenous vein prior to dosing and at 10min 20min 30min 1hr 3hr 5hr 7hr 24hr 48hr post dose.

Docetaxel used was powdered form to which we added Tween 80 and ethanol prior to dosing. Samples were collected in December of 2002 and not run until a year or more later.

Samples were spun, the plasma removed and then stored at -80° C until testing.

One animal died immediately following injection so there are:

17 animals 10 samples/animal Total: 170 samples

Repeat Experiment:

The differences between the first round and the repeat are:

- Sample Collection Times
0° 1min 5min 1hr 3hr 5hr 24hr 48hr post dosing
- Sample Storage
Samples were stored on ice until they could be processed (spun & plasma removed)
Some samples were tested within 4 months of collection
- Form of Docetaxel Used
Docetaxel used was in the commercial form rather than the powdered form used above

All 18 survived so there are:

18 animals 8 samples /animal Total: 144 samples

SJW/Docetaxel Pharmacokinetics

F344 rats

Diets from Harlan Teklad

6 animals on Control Diet

6 animals on SJW Diet (control diet plus 0.4% SJW. This is custom made for us and we provide the SJW)

Animals remained on diets for 2 weeks prior to receiving a 7mg/kg dose of docetaxel i.v. A heparinized blood sample was collected via saphenous vein prior to dosing and at 10 min 20min 30min 1hr 3hr 5hr 7hr. 24hr. 48hr post injection.

Docetaxel used was the powdered form to which we added Tween 80 and ethanol before dosing

Samples were centrifuged and the plasma was drawn off and stored at -80° C until testing.

Samples were collected in March and April of 2003 and not tested until now.

1 animal died immediately post dosing so there are:

11 animals 10 samples/animal Total: 110 samples

Repeat Experiment:

The differences between the first round and the repeat are:

- Sample Collection Times
0° 1min 5min 1hr 3hr 5hr 24hr 48hr
- Sample Storage

Samples were stored on ice until they could be processed
Some samples were tested within 4 months

➤ Form of Docetaxel Used

Commercial Form - not powdered form used above

➤ Dose:

10mg/kg instead of 7mg/kg

All 12 survived so there are:

12 animals 8 samples/animal Total: 96 samples

f. Repeat pharmacokinetic studies with different doses. Because of difficulties in detecting early time points, we have increased the dose of doxorubicin from 5 mg/kg to 7.5 mg/kg, and docetaxel from 7 mg/kg to 10 mg/kg.

Task 2. Measure the effects of supplementation with St. John's wort and vitamin E on the toxicity of cyclophosphamide, docetaxel and doxorubicin.

- a. Maintain 3 groups of rats on diet alone, or diet plus St John's wort or vitamin E.
 - b. Administer chemotherapeutic drugs in LD50 doses.
 - c. Observe for toxicity and collect blood samples.
 - d. Analyze blood samples for evidence of hematologic, renal, hepatic and cardiac toxicity.
 - e. Analyze toxicity data.
 - f. Repeat toxicological studies with different doses.
-
- i. As reported previously, weanling female Fisher 344 rats were maintained on the same diets described in Task 1; that is, cereal-based or supplemented with a low dose (50 I.U./kg) or a high dose of vitamin E (750 I.U./kg). The rats grew at the same rate on all 3 diets.
 - ii. As reported previously, after 8 weeks the rats were injected with increasing doses of doxorubicin. The LD50 of doxorubicin was approximately the same for all 3 diets (12.7-13.2 mg/kg) and there was no dose-response relationship for vitamin E.
 - iii. As reported previously, after 8 weeks the rats were injected with increasing doses of docetaxel. The LD50 of docetaxel was approximately the same (18 mg/kg) for all 3 diets. There was no significant difference in weight loss or measurements of hematocrit and white blood cell counts at Days 4, 9 and 14 following injection among the dietary groups.
 - iv. As reported previously, rats were maintained on either a control diet or the same diet supplemented with 0.4% St. John's wort, as described in Task 1. After 2 weeks, the rats were injected with increasing doses of doxorubicin. There were no striking differences in LD50, weight loss or hematologic toxicity between the two dietary groups.
 - v. Rats were maintained on either a control diet or the same diet supplemented with 0.4% St. John's wort and injected with increasing doses of docetaxel. As shown in Figure 2, the LD50 in the control diet animals was similar to the LD50 in the vitamin E experiment. However, the SJW data were very unusual and bear repeating.

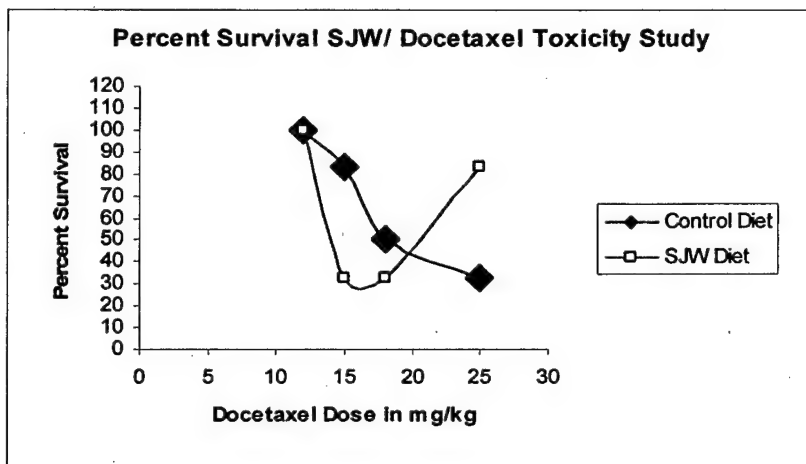
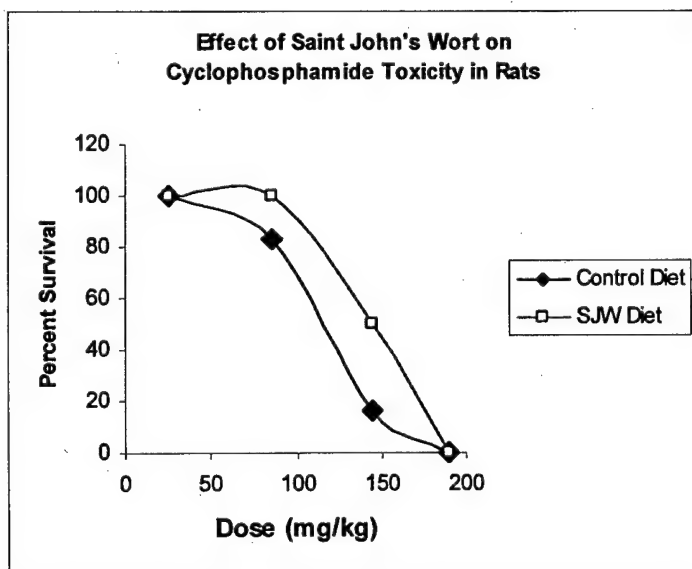


Figure 2. Effect of St. John's wort on survival after docetaxel.

vi. Rats were maintained on the control or St. John's wort diet and treated with increasing doses of cyclophosphamide (25, 85, 144 and 190 mg/kg). As seen in Figure 3, the rats fed St. John's wort had a higher LD50, suggesting that this herb decreased the toxicity of cyclophosphamide.

Figure 3. Effect of St. John's wort on cyclophosphamide toxicity.



vii. Rats were maintained on the control diet or diets supplemented with vitamin E for 8 weeks. They were then injected with increasing doses of cyclophosphamide (25, 85, 144 and 200 mg/kg).. As shown in Figure 4, below, there was no striking effect of vitamin E on cyclophosphamide toxicity.

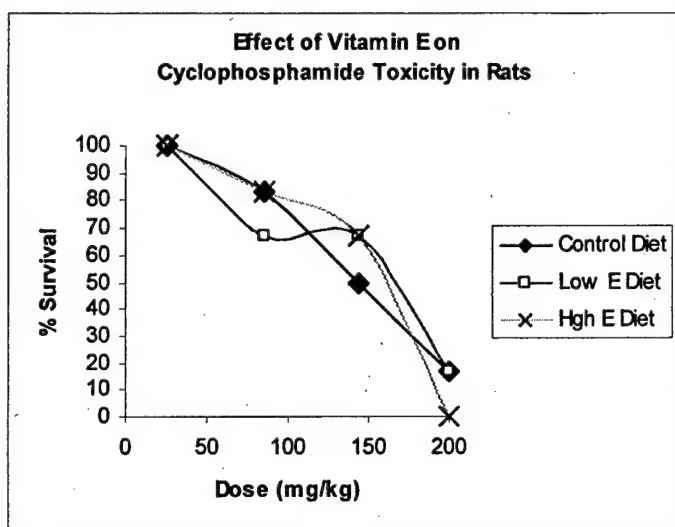


Figure 4. Effect of vitamin E on cyclophosphamide toxicity in rats.

Task 3. Study the mechanisms of nutraceutical-chemotherapeutic drug interactions.

- Measure hepatic p450 activity where appropriate.
- Measure hepatic P-glycoprotein expression where appropriate.
- Measure glutathione S-transferase activity in liver samples where appropriate.
- Collate and analyze data.

As previously reported, the possible modulatory effect of vitamin E on mitochondrial genetic damage caused by doxorubicin in rat heart and liver was studied. The technique was described in detail in the manuscript appended to last year's annual report. This manuscript is now in press (7). The data suggested that doxorubicin did not increase the number of deletions or copy number in cardiac or hepatic mitochondrial DNA, and that vitamin E supplementation does not modulate mitochondrial DNA damage.

During the past year we also measured the effect of vitamin E levels on mitochondrial DNA damage in rat liver caused by docetaxel. The Relative Expression of mitochondrial deletions was: Control, 1.00; Low E, 1.08; High E, 1.00. The Relative Expression of mitochondrial copy number was: Control, 1.00; Low E, 1.34; High E, 1.10. Therefore we conclude that dietary vitamin E levels do not modulate the mitochondrial DNA damage caused by docetaxel.

During the past year we completed a study of the effect of vitamin B12, folate and dietary supplements on breast cancer chemotherapy-induced mucositis and neutropenia in women. This work was started under a previous DOD grant (DAMD17-981-8345) and completed during this grant period. The results of this study, currently in press in *CANCER*, are directly relevant to the current grant. The full manuscript is appended.

BACKGROUND. Although patients with cancer frequently use dietary supplements, the effect of these agents on chemotherapy is unclear. Therefore we investigated the influence of vitamin B₁₂, folate and nutritional supplements on chemotherapy-induced toxicity.

METHODS. Women with breast cancer were asked to complete a questionnaire recording their use of dietary supplements. Blood samples were obtained for serum vitamin B₁₂ and folate levels before and after the first cycle of chemotherapy and for weekly complete blood counts. Toxicity was evaluated by absolute neutrophil counts and by the frequency and severity of oral mucositis.

RESULTS. Of 49 women who submitted questionnaires, 35 (71%) took a total of 165 supplements. Compared to a prior study in 1990, there was a dramatic increase in serum folate levels. Initial neutrophil count, but not type of chemotherapy, patient age or serum vitamin B₁₂ level was predictive of nadir absolute neutropenia and the drop from initial neutrophil count to nadir (Nfall). After adjusting for initial neutrophil count, Nfall was less in women taking supplements versus no supplements ($p=.01$), and in those taking multivitamins ($p=.01$) or vitamin E ($p=.03$). Women with serum folic acid levels <20 ng/ml had a smaller drop in neutrophil count after chemotherapy than those with higher folate levels ($p=.04$). No significant effect of initial neutrophil count, nadir neutrophil count, Nfall, age, vitamin B₁₂ or folate level on oral mucositis was found.

CONCLUSIONS. The drop in neutrophil count caused by cancer chemotherapy may be ameliorated by dietary supplementation with a multivitamin or vitamin E but is increased in association with high serum folate levels.

KEY RESEARCH ACCOMPLISHMENTS DURING THE PAST YEAR

- High performance liquid chromatography (HPLC) methodology was adapted for the determination of docetaxel.
- Samples have been collected for pharmacokinetic studies of docetaxel in control rats and in animals supplemented with vitamin E or St. John's wort. These samples currently are being analyzed.
- Dietary supplementation with St. John's wort resulted in an unusual toxicity profile for docetaxel.
- Dietary supplementation with St. John's wort increased the LD50 for cyclophosphamide, suggesting that this nutraceutical may decrease the toxicity of cyclophosphamide.
- Dietary supplementation with 2 levels of vitamin E had no effect on the toxicity of cyclophosphamide.
- Vitamin E supplementation did not modify hepatic mitochondrial DNA changes caused by docetaxel.
- In women with breast cancer, the drop in neutrophil count after chemotherapy was less in women taking supplements versus no supplements ($p=.01$), and in those taking multivitamins ($p=.01$) or vitamin E ($p=.03$). Women with serum folic acid levels <20 ng/ml had a smaller drop in neutrophil count after chemotherapy than those with higher folate levels ($p=.04$).

REPORTABLE OUTCOMES

Publications supported by this award:

Branda, R.F., Naud, S.J., Brooks, E.M., Chen, Z., Muss, H. The effect of vitamin B₁₂, folate and dietary supplements on breast cancer chemotherapy-induced mucositis and neutropenia.. *CANCER*, in press.

CONCLUSIONS

The experiments described in this Annual Report analyze the relationship between dietary supplements and chemotherapeutic drugs used to treat patients with breast cancer. Herbal medicines and dietary supplements are used frequently by patients with cancer, but there is a paucity of information on their interactions with prescribed drugs. Since a majority of women with breast cancer are taking dietary supplements, there is a pressing need to better understand the effects of these supplements on cancer chemotherapy. Our studies in rats suggest that even relatively high doses of vitamin E do not adversely affect the toxicity of doxorubicin, docetaxel, or cyclophosphamide. On the other hand, studies in women with breast cancer indicate that dietary supplementation with either a multivitamin or with vitamin E ameliorates the degree of neutropenia after combination chemotherapy. Our studies in rats suggest that St. John's wort may decrease the toxicity of cyclophosphamide. Further studies will help to determine which important interactions occur between these nutrients and cancer chemotherapeutic agents.

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**THE EFFECT OF VITAMIN B₁₂ , FOLATE AND DIETARY SUPPLEMENTS ON
BREAST CANCER CHEMOTHERAPY-INDUCED MUCOSITIS AND NEUTROPENIA**

Running Title: Diet Supplements and Drug Toxicity

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ABSTRACT

BACKGROUND. Although patients with cancer frequently use dietary supplements, the effect of these agents on chemotherapy is unclear. Therefore we investigated the influence of vitamin B₁₂, folate and nutritional supplements on chemotherapy-induced toxicity.

METHODS. Women with breast cancer were asked to complete a questionnaire recording their use of dietary supplements. Blood samples were obtained for serum vitamin B₁₂ and folate levels before and after the first cycle of chemotherapy and for weekly complete blood counts. Toxicity was evaluated by absolute neutrophil counts and by the frequency and severity of oral mucositis.

RESULTS. Of 49 women who submitted questionnaires, 35 (71%) took a total of 165 supplements. Compared to a prior study in 1990, there was a dramatic increase in serum folate levels. Initial neutrophil count, but not type of chemotherapy, patient age or serum vitamin B₁₂ level was predictive of nadir absolute neutropenia and the drop from initial neutrophil count to nadir (Nfall). After adjusting for initial neutrophil count, Nfall was less in women taking supplements versus no supplements ($p=.01$), and in those taking multivitamins ($p=.01$) or vitamin E ($p=.03$). Women with serum folic acid levels <20 ng/ml had a smaller drop in neutrophil count after chemotherapy than those with higher folate levels ($p=.04$). No significant effect of initial neutrophil count, nadir neutrophil count, Nfall, age, vitamin B₁₂ or folate level on oral mucositis was found.

CONCLUSIONS. The drop in neutrophil count caused by cancer chemotherapy may be ameliorated by dietary supplementation with a multivitamin or vitamin E but is increased in association with high serum folate levels.

Key Words: diet, folate, vitamins, chemotherapy, toxicity

INTRODUCTION

Relatively little is known regarding the effects of dietary components and nutritional supplements on cancer chemotherapy (1). Nevertheless, many patients alter their diets and begin taking nutritional supplements and vitamins after they are diagnosed with cancer, and this practice appears to be increasing (2,3). In a seminal 1984 paper, Dr. Barrie Cassileth reported that 37% of cancer patients on conventional treatment also used diet therapy and 30% used megavitamin therapy (4). More recently Cassileth reviewed 26 surveys of cancer patients from 13 countries, including 5 performed in the United States, and found a 31% average prevalence of use of complementary/alternative cancer medicine (5). However, a 1999 survey of 8 clinics at the M.D. Anderson Cancer Center found that 83% of patients used complementary/alternative therapy, and that the most frequently used approach (62%) was herbal and/or vitamin supplementation (6).

Our interest in this area began with studies of the effect of folic acid supplementation on chemotherapy in breast cancer. In 1998 we reported that nutritional folate status influenced the efficacy and toxicity of chemotherapy in rats (7). We found that cyclophosphamide and doxorubicin were 183% and 244% as effective, respectively, against a rat mammary tumor, with less host toxicity, in folate-supplemented rats compared to folate-deficient animals (7). Exploratory studies in our laboratory of the mechanism underlying this interaction of folate metabolism and cancer chemotherapy suggested a previously under-appreciated relationship between folate status and glutathione levels (8). Glutathione levels are an established determinant of chemotherapy toxicity (9). More recent studies in rats provided additional evidence for the influence of diet on cancer chemotherapy. We found that rats on a cereal-based

diet were far more resistant to the toxic effects of cyclophosphamide than rats on a purified diet (8).

Following the FDA-mandated fortification of cereal foods with folic acid, there has been a remarkable shift toward high blood folic acid levels in the general population (10,11). While our animal studies suggest that elevated folic acid levels may be beneficial to patients undergoing chemotherapy, the relevance of these studies to humans is uncertain. Recently, Sellers and colleagues found that high-folate diets did not have a significant adverse effect on survival after chemotherapy for breast cancer (12). In this report we describe the influence of vitamin B₁₂ and folate status and the use of dietary supplements on the toxicity of chemotherapy in a pilot study of women with breast cancer.

MATERIALS AND METHODS

Population Studied. Women with histologically proven breast cancer were asked to participate in this study. After informed consent was obtained, following procedures approved by the University of Vermont Committee on Human Research, blood samples were obtained and the women completed a questionnaire. Any woman with breast cancer regardless of stage was eligible.

Sample Collection. Weekly blood counts (hemoglobin, hematocrit, white blood cell count with differential, and platelet count) were obtained during the first cycle of chemotherapy, typically 3 or 4 weeks. Serum was collected before the first and second cycles of chemotherapy and cryopreserved in the dark for subsequent analysis of vitamin levels.

Assays of Vitamin Levels. Serum vitamin B₁₂ and folate levels were measured using the Quantaphase II B₁₂ and Folate Radioassay (Bio-Rad Diagnostics Group, Hercules, CA 94547).

The assays were performed by combining a serum sample with vitamin B₁₂ (⁵⁷Co) and/or folate (¹²⁵I) in a solution containing dithiothreitol and cyanide. The mixture was combined with immobilized, affinity-purified porcine intrinsic factor and folate binding protein. Labeled and unlabeled vitamins binding to the immobilized binding proteins were concentrated in a pellet, and the radioactivity of the pellet was counted. Standard curves were prepared using vitamin B₁₂ and folate standards in a human serum albumin base.

Toxicity Assessment. Neutropenia was analyzed in three ways. The first method was to measure neutrophil decrease (Nfall) as the difference between the neutrophil count immediately before chemotherapy and the nadir count during the first cycle (13). The second method was to identify the nadir absolute neutrophil count. The third method was to categorize neutrophils by grade, with grade 4 = $\leq 0.100 \times 10^9 / L$, 3 = 0.101 to $0.500 \times 10^9 / L$, 2 = 0.501 to $1.000 \times 10^9 / L$, and 0-1 = $> 1.000 \times 10^9 / L$. The nadir absolute neutrophil count was highly correlated with the neutrophil count 2 weeks after treatment ($r = 0.94$) (Figure 1). Only 2 patients were admitted to the hospital for evaluation and treatment of febrile neutropenia.

The patient's physician was asked to grade the degree of oral mucositis semi-quantitatively by a modification of the National Cancer Institute common toxicity criteria (version 2.0) (Grade 0=none; Grade 1=mild; painless ulcers, erythema, or mild soreness in the absence of lesions; Grade 2=moderate; painful erythema, edema or ulcers but can eat or swallow; Grades 3 and 4=severe; painful erythema, edema or ulcers preventing swallowing or requiring hydration or parenteral support or requiring intubation).

Statistical Analysis. The statistical analyses were run using SAS version 6.12 (SAS Institute Inc., Cary, NC: STAT Institute Inc., copyright 1989-1996). Continuous outcomes were analyzed using t-test, regression, ANOVA or ANCOVA. The folate values and initial and nadir

neutrophil counts had skewed distributions so the natural log transformation was used. Binomial outcomes were analyzed with logistic regression. If both variables could be considered as being ordinal, then the gamma statistic was used. In 7 patients, there was no blood sample collected before the first cycle of chemotherapy for vitamin assay, but a sample was obtained prior to the second cycle. Analysis indicated that the vitamin B₁₂ and folic acid values from these samples could be substituted for statistical purposes.

RESULTS

Seventy-nine patients were enrolled in this study; of these, 68 patients met the exclusion criteria of having an initial neutrophil count and at least 2 neutrophil counts in the first 3 weeks (Table 1). Their ages were a mean of 48.1 ± 9.3 years with a range of 30-75 years. Fifty-four patients received doxorubicin/cyclophosphamide, 3 received doxorubicin with docetaxel, 6 patients received cyclophosphamide, methotrexate and 5-fluorouracil, 2 received single agent doxorubicin, and 3 received single agent taxane (2 paclitaxel, 1 docetaxel) (Table 1).

Supplement questionnaire. Forty-nine women who met the above exclusion criteria submitted questionnaires that listed their dietary supplement use at the time of enrollment (Table 2). Of these, 14 (29%) took no supplements. The remaining 35 women (71%) took a total of 165 supplements. Usage ranged from a single agent (8 patients) to 20 different substances taken daily by one patient. The mean number of supplementation substances taken was 3 and the median was 2. Twenty patients (41%) took supplements other than vitamins and minerals, usually complex mixtures of herbal extracts. Thirty-two patients took 1 or more vitamin supplements; 17 of the 32 also took mineral supplements – most commonly calcium (53%). There were no differences in initial neutrophil count, nadir or Nfall between the group taking

only vitamin supplements and the patients taking both mineral and vitamin supplements (t-test, $p>.40$). Therefore, mineral supplements did not add any beneficial effect independent of vitamins.

Comparisons were made between the no-supplement group and the subjects taking any kind of supplement. After adjusting for initial neutrophil count, the supplement group had a significantly lower Nfall than the no-supplement group (ANCOVA $p=.01$), but the difference in the nadir count was not significant. There were three vitamin supplements taken by more than 10 of the subjects, namely multivitamins (47%), and vitamins C (22%) and E (37%). None of these was significantly associated with nadir count ($.09<p<.28$), but multivitamins and vitamin E were significantly associated with Nfall ($p=.01$ and $.03$ respectively) (Figure 2). The significant associations corresponded to a smaller Nfall that decreased by 625 to 750 $\times 10^9/L$ less for the supplement group (after adjusting for differences in initial neutrophil count). That is, patients taking these supplemental vitamins had a smaller drop in absolute neutrophil count after chemotherapy than women who did not take them. This pattern of results was the same when the analyses were repeated for the subset of subjects who had doxorubicin/cyclophosphamide treatment (11 in the no-supplement group, 29 taking supplements). The differences in Nfall were larger in magnitude (742 to 909 $\times 10^9/L$). There was a suggestion of an interaction of supplement use, but because of the small numbers, no clear picture emerged. Multivitamin or vitamin E supplement use versus no supplement use was associated with more grade 0-1 (30%, 22% and 7%, respectively) and less grade 3 neutropenia (26%, 33% and 57%, respectively).

Serum levels. Serum vitamin B₁₂ and folate levels were measured at the beginning and/or the end of the first cycle of chemotherapy in 63 patients. The first vitamin B₁₂ level was 526 ± 330 pg/ml (mean \pm SD), and the second was 628 ± 560 pg/ml. The two vitamin B₁₂ levels were

correlated ($r=0.64$, $p<0.0001$). The mean \pm SD for the first and second folate levels were 17.8 ± 9.6 ng/ml and 18.6 ± 9.9 ng/ml, respectively. The two folate levels were correlated as well (0.56 , $P = 0.002$).

In 1990 we measured serum folate levels in 47 women with breast cancer from the same community and socio-economic group using a similar assay system (14,15). Table 3 shows the results of that study compared with folate levels observed in the current study. It can be seen that there has been a dramatic shift toward higher serum folate levels in this population over the past decade.

Regression analyses were performed with nadir absolute neutrophil count and Nfall as outcomes and the initial absolute neutrophil count, patient age, the serum vitamin B₁₂ level, the serum folate level and the type of chemotherapy (dichotomous variables: cyclophosphamide/doxorubicin or doxorubicin alone versus any other chemotherapy--- 5-fluorouracil, docetaxel, methotrexate or paclitaxel) separately as predictors. The only significant predictor of both outcomes was the initial neutrophil count ($p<0.01$). The type of chemotherapy was not significant for either outcome ($p>0.20$). Repeating the regressions when limiting the analysis to the subset of patients who received cyclophosphamide and doxorubicin produced the same pattern of results: only the initial neutrophil count was significant. The predictors were transformed into categorical variables (age: <40 , 40 to <50 , >50 years old), vitamin B₁₂ (<400 , 400 to <800 , >800 pg/ml) and folate (20 or less, >20 ng/ml). Because the initial neutrophil count was significant in the regressions, it was included in the ANCOVA models as a covariate. Neither the type of chemotherapy, age, nor vitamin B₁₂ level was a significant predictor for nadir neutrophil count or Nfall ($p>0.15$). Folate category was significant ($p=0.04$) for Nfall but not for nadir count ($p=0.11$). The model estimates that the Nfall is $426 \times 10^9/L$ lower for the subjects

with folate levels less than 20 ng/ml, after adjusting for initial neutrophil count (Figure 2). For the doxorubicin/cyclophosphamide subset, the significance of the folate group declined somewhat ($p=0.06$). Thus patients with relatively lower serum folate levels had a smaller decrease in neutrophil count after chemotherapy compared to women with higher folate levels (Figure 2).

Mucositis. The distribution of oral mucositis was Grade 0=51 patients, Grade 1=11 patients, Grades 2 and 3=6 patients, and 4=0 patients. Logistic regression was used to evaluate which variables can be used to predict mucositis (0 versus any value). Initial neutrophil count, nadir neutrophil count, Nfall, initial vitamin B₁₂ and folate levels, and age were not found to be significant (all $p>0.10$). These predictors were then tested as categorical variables, and no significant effect was found. There was no effect of supplementation with multivitamins or vitamin E on the severity of mucositis.

DISCUSSION

Our study in women with breast cancer confirms prior investigations that found that the majority of cancer patients are taking one or more dietary supplements or herbal remedies (2-6). The most commonly used agents in our study were multivitamins, vitamin E and calcium, but some patients took as many as 20 different substances. Because vitamins and other nutritional supplements are natural products, patients often consider them less toxic alternatives or additions to conventional cancer chemotherapy. However, there is growing evidence that these dietary supplements can mimic, increase or decrease the effects of drugs (16,17). Unfortunately there are relatively few studies of the effects of alternative medicine, which often includes dietary supplements and herbal medicines, on cancer chemotherapy, and many of these give conflicting

results (1). For example, a few small trials of antioxidant supplements in patients with breast, lung and squamous cancer have hinted at a survival benefit, but comparisons were with historical controls (18,19). Vitamin users with non-small cell lung cancer had a longer median survival compared to nonusers, but the number of subjects was too small to identify the specific micronutrient responsible for the beneficial effect (20). Conversely, a comparison of women with breast cancer who were prescribed mega-doses of beta-carotene, vitamin C, niacin, selenium, coenzyme Q10 and zinc with matched controls found that breast-cancer specific survival and disease-free survival times were shorter for the vitamin/mineral treated group (21). Supplementation with β -carotene or α -tocopherol did not alter the mortality rate from pancreatic cancer (22). Burstein and colleagues reported that the use of alternative medicine by women with breast cancer was a marker of greater psychosocial distress and worse quality of life (3). In this study, women who received chemotherapy were more likely to begin using alternative medicine (3). Finally, a recently published report showed striking reductions in the active metabolite of irinotecan in patients who were taking St. John's Wort (23). Thus the beneficial effect of vitamin supplementation or herbal remedies, if any, on the efficacy of chemotherapy and on patient survival remains uncertain.

Dietary supplementation with vitamins, particularly vitamin E, has been recommended to decrease the toxicity of chemotherapy. Since reactive oxidant species have been implicated in doxorubicin-induced cardiomyopathy, bleomycin-induced pulmonary fibrosis, and cisplatin-induced neuropathy and nephrotoxicity, antioxidants have been used to reduce or prevent these side effects (24 and reviewed in 25). Several studies support the possibility that vitamin E supplementation may increase the efficacy and reduce the toxicity of cancer chemotherapy, particularly of doxorubicin-containing regimens (25). There has been little or no evidence,

however, to suggest that vitamin E supplementation influenced chemotherapy-induced myelosuppression, mucositis, nausea or vomiting (25). A recent review of the literature by Seifried and colleagues concluded that current knowledge makes it premature to generalize and make specific recommendations about antioxidant use during cancer chemotherapy (26). In our study, we found that vitamin E influenced myelosuppression caused by chemotherapy by reducing the drop in absolute neutrophil count from initial to nadir levels. It did not, however, affect the nadir counts.

Oral mucositis is a distressing side-effect of chemotherapy and contributes to the morbidity and mortality of high-dose chemotherapy (27). A peak oral mucositis score such as the one used in this study is a useful index of gastrointestinal toxicity and correlates with clinically significant events such as bacteremia (27). In prior studies, diagnosis, treatment protocol, rate of neutrophil recovery, and patient age were found to influence the severity of mucositis (27). Oral L-glutamine has been shown to decrease the duration and severity of oral mucositis, particularly when used with chemotherapy regimens that contain doxorubicin or methotrexate (28). In our study, initial neutrophil count, Nfall, nadir absolute neutrophil count and serum vitamin B₁₂ and folate levels were not significantly related to the risk of developing mucositis. In contrast to the results of a prior study (27), we did not find that younger patients were more likely to experience oral mucositis.

In view of the remarkable overall increase in blood folate levels both in the general population (10,11) and in our patients with breast cancer, it was of interest to determine if elevated folate levels modulated myelosuppression by chemotherapy. Our investigations in rats suggested that the interaction between folate levels and cancer chemotherapy is complex. Initial studies indicated that cyclophosphamide was less effective at inhibiting the growth of a rat

mammary tumor in folate-deficient rats (7). In addition, toxicity from cyclophosphamide was increased in folate-deficient rats and ameliorated in folate-supplemented animals compared to rats that ingested the standard amount of folic acid (7). The toxicity of 5-fluorouracil was significantly greater in folate-deficient rats than in folate-replete or supplemented rats, but there was no relationship between folate status and doxorubicin toxicity (7). Thus the effects of folate status on toxicity varied with the chemotherapeutic agent. A subsequent study indicated that the chemotherapy schedule also may influence the interaction. When the drugs were given as a single bolus instead of in divided doses, high folate rats developed more severe anemia, azotemia and leukopenia after 5-fluorouracil but no different toxicity with cyclophosphamide (8). In the case of both drugs, rats on a cereal-based diet were more resistant to toxicity than animals on a purified diet. The cereal-based diet appeared to protect against severe renal damage caused by the combination of 5-fluorouracil and a purified diet supplemented with folic acid (8). The women in the current study were predominantly treated with bolus cyclophosphamide and doxorubicin. We found no significant association between serum vitamin B₁₂ level and neutropenia. Serum folic acid levels influenced the Nfall but not the absolute neutrophil nadir. Women with serum folate levels <20 ng/ml had a smaller decrease from initial to nadir absolute neutrophil counts than women with higher folate levels. There were too few women treated with 5-fluorouracil to determine if chronically high levels of folic acid lead to renal damage or more severe neutropenia with this drug (5 of 6 patients had serum folate levels <20 ng/ml).

The major limitation of this study is its small sample size. The trial was designed as a pilot study in a single institution to identify nutritional components, particularly vitamin B₁₂ and folate, that might affect chemotherapy-induced toxicity. There is sufficient power to indicate that blood levels of vitamin B₁₂ are unlikely to have a major effect on neutropenia or mucositis,

but relatively small effects may have been missed. Given the sizeable number and diversity of dietary supplements taken by these patients, a much larger clinical trial will be required to measure the effect on toxicity of any but the most commonly used specific agents. A second limitation of the study is the heterogeneous chemotherapeutic regimens that were used. Although the majority of the patients received doxorubicin/cyclophosphamide as adjuvant chemotherapy, and 87% of the patients received doxorubicin either as a single agent or in combination, it is likely that some drug regimens are more likely to cause neutropenia or mucositis than other drugs. Finally, the study included patients with both newly diagnosed and advanced breast cancer. It is possible that patients who were previously treated may have been more susceptible to neutropenia or mucositis.

In conclusion, we found a high prevalence of dietary supplement use in women with breast cancer. As is the case in the general population (10,11), many of these women had elevated blood folate levels. There was evidence that the decrease in neutrophil count after chemotherapy was ameliorated by ingestion of multivitamins or vitamin E. However, women with high serum folate levels were more likely to have greater drops in their neutrophil counts after chemotherapy. In view of the small sample size in this pilot study, these results will need to be confirmed in a larger number of patients.

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LEGENDS

Figure 1. Total white blood cell count (stipled bars) and absolute neutrophil counts (open bars) measured before and weekly during the first course of cancer chemotherapy in 68 women with breast cancer. The nadir absolute neutrophil count was highly correlated with the neutrophil count 2 weeks after treatment ($r = 0.94$). Error bars represent standard error of the mean.

Figure 2. The effect of vitamin supplementation and serum folic acid levels on neutropenia following cancer chemotherapy. Neutropenia was assessed by measuring the difference between the initial absolute neutrophil count and the nadir count (Nfall). Brackets show the standard error of the mean. There were 14 patients who took no supplements, 18 who took vitamin E, and 23 who took supplemental multivitamins. There were 39 women with serum folic acid levels <20 ng/ml and 24 with levels >20 ng/ml. The comparisons were significantly different ($p=.03$ for vitamin E, $p=.01$ for multivitamins, and $p=.04$ for folic acid).

Table 1. Number of patients treated with chemotherapy regimens in the adjuvant, neoadjuvant or metastatic disease settings.

Total patients (number)	68	
Adjuvant Chemotherapy	56	
doxorubicin/cyclophosphamide (600/60 mg/m ²)		47
cyclophosphamide/methotrexate/5-fluorouracil (600/40/600 mg/m ²)		6
doxorubicin/docetaxel (45/75 mg/m ²)		2
doxorubicin (60 mg/m ²)		1
Neoadjuvant Chemotherapy	6	
doxorubicin/cyclophosphamide (600/60 mg/m ²)		6
Metastatic Disease	6	
doxorubicin/cyclophosphamide (600/60 mg/m ²)		1
doxorubicin/docetaxel (45/75 mg/m ²)		1
doxorubicin (75 mg/m ²)		1
paclitaxel (175 mg/m ²)		2
docetaxel (40 mg/m ² weekly x3)		1

Table 2. Dietary supplement use by women with breast cancer.

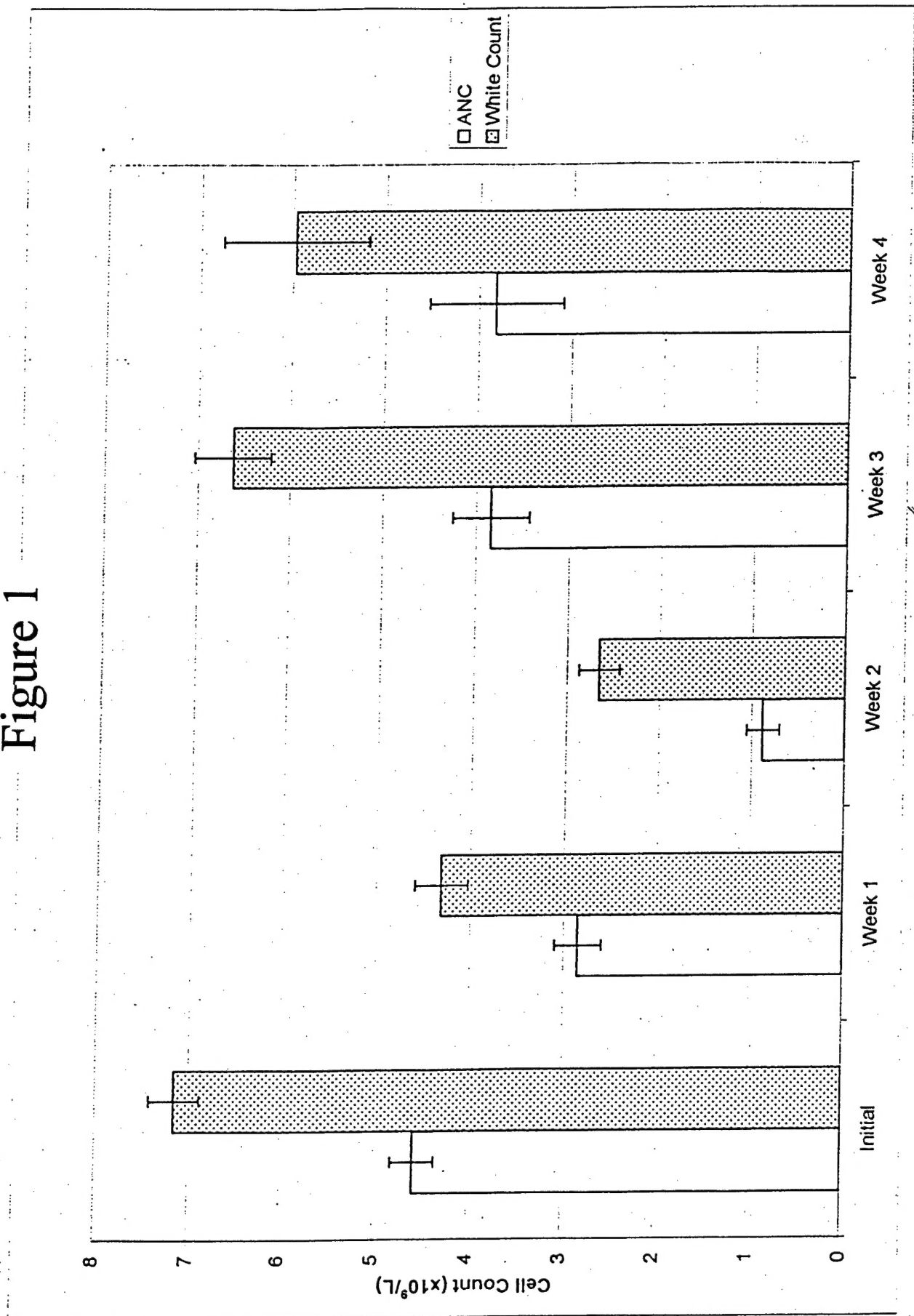
Supplement Use	N*	%						
None	14	29						
Single Supplement	8	16						
2-5 Supplements	18	37						
6-10 Supplements	5	10						
>10 Supplements	4	8						
<u>Supplements Taken</u>								
<u>Vitamins</u>	<u>N</u>	<u>%</u>	<u>Minerals</u>	<u>N</u>	<u>%</u>	<u>Nutraceuticals</u>	<u>N</u>	<u>%</u>
Multivitamin	23	47	Mineral complex	3	6	Ginko	7	12
Vitamin A	5	10	Calcium	13	27	Echinacea	3	6
Folic acid	3	6	Iron	1	2	Co-enzyme Q	8	14
Vitamin B ₁₂	2	4	Magnesium	2	4	Pectin	2	3
Vitamin B complex	3	6	Selenium	3	6	Glutamine	3	5
Vitamin C	11	22	Zinc	1	2	Other	18	37
Vitamin D	3	6						
Vitamin E	18	37						

*N = number of patients

Table 3. Serum folate levels measured ten years apart in two different groups of women with breast cancer from the same community.

Year	1990		2000	
<u>Serum Folate Level</u>	<u>Number</u>	<u>%</u>	<u>Number</u>	<u>%</u>
Deficient (<3.0 ng/ml)	12	26	0	0
Indeterminate (3.1-6.0 ng/ml)	18	38	4	6
Sufficient (6.1-20 ng/ml)	17	36	35	56
High (>20 ng/ml)	0	0	24	38

Figure 1



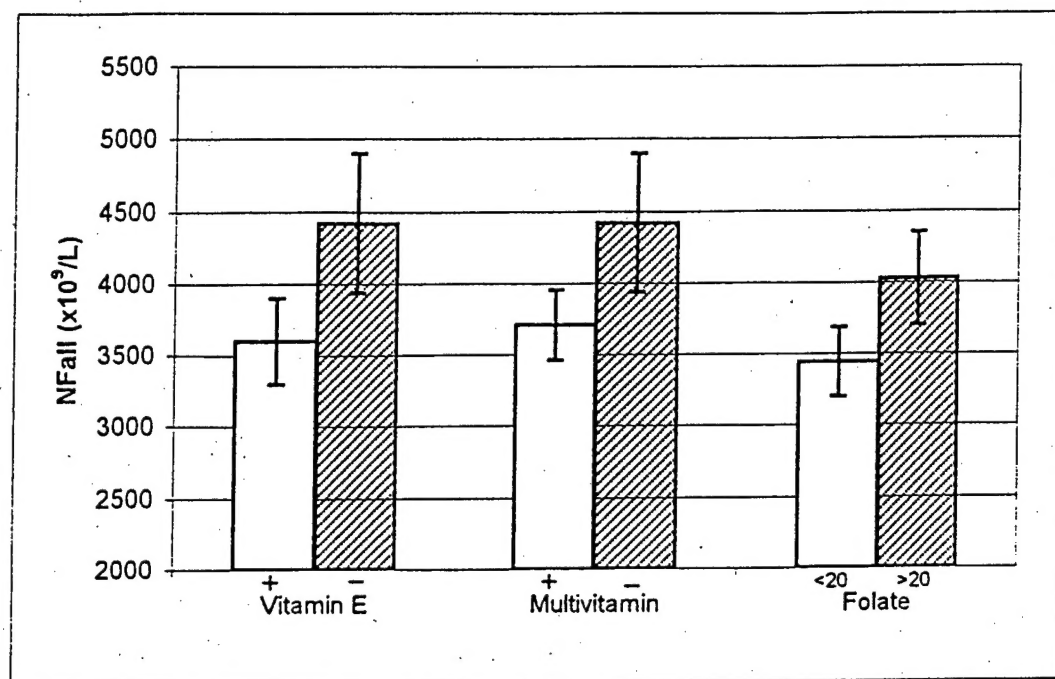


Figure 2